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Use of Polymerase Chain Reaction for Detection of Toxigenic *Vibrio cholerae* O1 Strains from the Latin American Cholera Epidemic

PATRICIA I. FIELDS, TANJA POPOVIC, KAYE WACHSMUTH, AND ØRJAN OLSVIK*

Enteric Diseases Branch, Division of Bacterial and Mycotic Diseases, National Center for Infectious Diseases, Centers for Disease Control, Atlanta, Georgia 30333

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In January 1991, an outbreak of cholera started in Peru and spread throughout most of Latin America within 8 months. As of March 1992, over 450,000 cases and approximately 4,000 deaths have been reported to the Pan American Health Organization. The causative organism is toxigenic *Vibrio cholerae* O1 of the El Tor biotype and is distinct from the U.S. Gulf Coast strains. A polymerase chain reaction (PCR) that amplifies a 564-bp fragment of the cholera toxin A subunit gene (*ctxA*) was used to identify toxigenic *V. cholerae* O1 strains. A total of 150 *V. cholerae* O1 isolates were tested. They were of unknown toxin status, were associated with recent outbreaks, and were isolated from patients, food, and water. One hundred forty isolates were found to be toxigenic both by PCR and the routine diagnostic enzyme-linked immunosorbent assay. Thirty-eight known toxigenic strains isolated worldwide from 1921 to 1991 were also positive in the PCR. A collection of 18 nontoxigenic *V. cholerae* O1 strains, 35 *Escherichia coli* heat-labile-enterotoxin-I-producing strains, 26 *Campylobacter* strains, and 8 strains of *Aeromonas hydrophila*, previously reported to produce cholera toxin-like toxin, were all negative in the *ctxA* PCR. We conclude that this PCR is a diagnostic method that specifically detects toxin genes in *V. cholerae* O1 strains in a reference laboratory. It is more rapid and less cumbersome than other diagnostic methods for detection of toxicity in these strains.

An outbreak of cholera began in Peru in January 1991 and has since spread to most countries in Latin America (5). As of March 1992, about 450,000 cases and 4,000 deaths in the Western Hemisphere have been reported to the Pan American Health Organization. Most of the cases were in Peru; Peru reported 375,000 cases and more than 3,000 deaths. Ecuador reported more than 50,000 cases and 800 deaths, and Colombia reported more than 13,000 cases and 200 deaths. Cases of cholera have also been reported from Guatemala, Mexico, Brazil, Chile, El Salvador, Honduras, Bolivia, Nicaragua, Venezuela, Panama, Argentina, Costa Rica, Belize, Surinam, and French Guiana. During this time, sporadic cases occurred in Canada and the United States, but all of these were associated with travel to countries with cholera endemics or with consumption of food from those countries (6, 7). The causative organism of this epidemic is *Vibrio cholerae* O1, El Tor biotype, Inaba serotype; however, the Ogawa serotype has been isolated from clusters of cases in Peru and Bolivia. The Latin American isolates are clearly distinguishable from strains of *V. cholerae* O1 isolated from cases of cholera occurring along the Gulf Coast of the United States during the last 10 years (4, 18).

Only strains of *V. cholerae* O1 that produce cholera toxin have been associated with epidemics and pandemics in the past; therefore, production of cholera toxin has become an important marker for identifying isolates with the potential to cause epidemics (9). Cholera toxin belongs to a family of related enterotoxins that consist of two polypeptides. The A subunit is responsible for adenylate cyclase activation in small intestinal epithelial cells, inducing the active secretion of water and salts, which causes the tremendous fluid loss associated with cholera. The B subunit consists of five identical elements responsible for binding to the epithelial cell surface receptor, GM_1. The genes expressing A and B subunits are designated *ctxA* and *ctxB*, respectively. They are expressed as a single transcriptional unit, and the structural genes overlap by one base (11).

Molecular epidemiologic approaches have been essential in documenting the spread of cholera throughout the Americas, and this technology has promise as the basis for a new generation of diagnostic assays for this increasingly important pathogen (1, 17, 18). In this article, we describe the development and evaluation of a polymerase chain reaction (PCR) that amplifies *ctxA*, and we present data generated from its use in the current outbreak of cholera in the Americas.

MATERIALS AND METHODS

Strains and templates. One hundred and forty-nine isolates of *V. cholerae* O1 found in investigations of outbreaks of cholera in 1991, mainly in Latin America and the United States, were studied (Table 1). In addition, a collection of 38 toxigenic and 18 nontoxigenic *V. cholerae* O1 strains isolated over the last 60 years were used as reference material (Tables 2 and 3). Thirty-five *Escherichia coli* strains of different serotypes and origins, previously shown to produce heat-labile enterotoxin (LT), eight *Aeromonas hydrophila* strains, and 26 *Campylobacter jejuni* and *Campylobacter coli* strains that have been reported to produce a cholera toxin-like toxin (3, 10, 14, 16) were studied for comparison. All strains are part of the culture collection in the Enteric Diseases Branch, Division of Bacterial and Mycotic Diseases, National Center for Infectious Diseases, Centers for Disease Control, Atlanta, Ga.

PCR. Routinely, bacterial colonies from an overnight cultivation on blood agar plates (Trypticase soy agar II

* Corresponding author.

TABLE 1. *V. cholerae* O1 El Tor strains isolated during 1991 outbreaks

Origin	Sample	Date (mo., day) first tested[a]	No. of strains	Serotype	PCR for *ctxA*
Peru	Human stool	Feb. 6	6	Inaba	+
Ecuador	Human stool	March 19	1	Inaba	+
Ecuador	Human stool	March 25	1	Inaba	+
Georgia	Human stool	April 11	1	Inaba	+
New Jersey	Human stool	April 20	4	Inaba	+
Peru	Human stool	June 3	5	Ogawa	+
Chile	Human stool	May 21	40	Inaba	+
New York	Human stool	May 22	1	Inaba	+
Mexico	Human stool	July 8	28	Inaba	+
Ecuador	Human stool	July 16	3	Inaba	+
Alabama	Oysters	July 16	4	Inaba	+
Alabama	Oysters	July 23	3	Inaba	+
Guatemala	Human stool	July 23	4	Inaba	+
Colombia	Human stool	July 23	2	Ogawa	+
Alabama	Oysters	July 26	4	Inaba	+
Alabama	Oysters	July 26	2	Inaba	−
Mexico	Human stool	Aug. 5	6	Inaba	+
Ecuador	Conches	Aug. 5	1	Inaba	+
Guatemala	River water	Aug. 20	1	Inaba	−
El Salvador	Human stool	Aug. 22	1	Ogawa	+
Bolivia	Human stool	Aug. 29	1	Ogawa	+
Maryland	Coconut milk	Aug. 30	1	Ogawa	+
Peru	Seawater	Sept. 3	2	Inaba	+
Peru	Seawater	Sept. 3	3	Ogawa	+
El Salvador	Potable water	Sept. 11	1	Ogawa	−
Maryland	Coconut milk	Sept. 23	1	Ogawa	+
Romania	Human stool	Sept. 23	1	Ogawa	+
Romania	Human stool	Sept. 23	1	Ogawa	−
Alabama	Oysters	Sept. 25	2	Inaba	+
Louisiana	Moore swab	Sept. 26	2	Inaba	−
Hawaii	Human stool	Oct. 4	2	Ogawa	+
Alabama	Ballast water from a ship	Nov. 14	5	Inaba	+
El Salvador	Human stool	Dec. 19	1	Inaba	−
Bolivia	Water	Dec. 19	1	Ogawa	−
Venezuela	Human stool	Dec. 27	1	Inaba	+
Guatemala	Human stool	Dec. 27	1	Ogawa	+
Louisiana	Ballast water from a ship	Dec. 31	1	Inaba	+
Maryland	Human stool	Dec. 31	1	Inaba	+
El Salvador	Human stool	Dec. 31	3	Inaba	+

[a] Each date represent a different shipment of strains.

TABLE 2. Toxigenic *V. cholerae* O1 strains positive for *ctxA* by PCR

Strain	Biotype	Serotype	Origin	Year of isolation
75	Classical	Inaba	Japan	1921
569B	Classical	Inaba	Hong Kong	1940
H23337	Classical	Inaba	Dhaka, Bangladesh	1970
9060-79[a]	Classical	Ogawa	India	1949
E8260	El Tor	Inaba	Gilbert Island	1966
E8263	El Tor	Inaba	Ethiopia	1971
E8262	El Tor	Inaba	Algeria	1972
3242-73	El Tor	Inaba	Texas, U.S. Gulf Coast	1973
2538-86	El Tor	Inaba	Georgia, U.S. Gulf Coast	1976
2270-77	El Tor	Inaba	Australia	1977
2164-78	El Tor	Inaba	Louisiana, U.S. Gulf Coast	1978
2213-80	El Tor	Inaba	Cameroon	1980
2097-80	El Tor	Inaba	New Zealand	1980
1428-81	El Tor	Inaba	Texas, U.S. Gulf Coast	1981
2673-81	El Tor	Inaba	Saudi Arabia	1981
E1800-82	El Tor	Inaba	Truk, South Pacific	1982
776-83	El Tor	Inaba	Mexico	1983
2484-86	El Tor	Inaba	Thailand	1986
C7950	El Tor	Inaba	Hong Kong	1986
2463-88[b]	El Tor	Inaba	Australia	1988
C7958	El Tor	Inaba	Hong Kong	1989
C6610	El Tor	Inaba	Malawi	1990
C5631	El Tor	Inaba	Truk, South Pacific	1990
E9112	El Tor	Ogawa	Malaya, Asia	1963
E9121	El Tor	Ogawa	Iran	1965
E9956	El Tor	Ogawa	Philippines	1966
E8248	El Tor	Ogawa	Libya	1970
E8249	El Tor	Ogawa	Lebanon	1970
E8252	El Tor	Ogawa	Spain	1971
E8264	El Tor	Ogawa	Ivory Coast	1971
E8253	El Tor	Ogawa	Tunisia	1973
E8257	El Tor	Ogawa	Kuwait	1977
X25514	El Tor	Ogawa	Dhaka, Bangladesh	1982
912-84	El Tor	Ogawa	Mali	1984
2472-86	El Tor	Ogawa	Philippines	1986
2432-88	El Tor	Ogawa	Rwanda	1988
2459-90	El Tor	Ogawa	India	1990
2560-90	El Tor	Ogawa	Guam, South Pacific	1990

[a] ATCC 14035.
[b] First tested negative, then positive when retested.

[Becton Dickinson and Company, Cockeysville, Md.] with 5% sheep blood) were suspended in 0.5 ml of sterile water to a concentration of 10^5 to 10^6 organisms per ml, boiled for 30 min, and used as templates for the PCR. Additionally, PCR was also performed on organisms taken with a loop directly from the transport medium (tryptic soy agar or nutrient agar) when it arrived at the Centers for Disease Control. Two primers (CTX2, CGG GCA GAT TCT AGA CCT CCT G, positions 73 to 94 [11]; CTX3, CGA TGA TCT TGG AGC ATT CCC AC, positions 614 to 636 [11]) that amplify a 564-bp region of the *ctxA* gene were used. The PCR was carried out in 0.5-ml microcentrifuge tubes, using a 50-μl reaction mixture consisting of 38.75 μl of sterile water, 5 μl of 10× PCR buffer (100 mM Tris-HCl [pH 8.3], 500 mM KCl; 15 mM $MgCl_2$, 0.1% [wt/vol] gelatin), 4 μl of deoxynucleoside triphosphates (2.5 mM [each] dATP, dTTP, dGDP, and dCTP), 0.5 μl of each primer (100 μM), 1 μl of template, and 0.25 μl of *Taq* polymerase (The Perkin-Elmer Corp., Norwalk, Conn.). The solution was overlaid with 1 drop of mineral oil before being placed in a thermocycler. Thermocyclers from the Perkin-Elmer Corp. (model 480), Stratagene (model SCS-96; Stratagene, La Jolla, Calif.), and M. J. Research, Inc. (Watertown, Mass.) were used interchangeably. The cycling conditions were as follows: preincubation at 95°C for 5 min; 25 cycles of 1 min at 95°C, 1 min at 60°C, and 1 min at 72°C; and a final incubation at 72°C for 10 min. Annealing temperatures of 50 and 55°C were used in some experiments. The amplicons were electrophoresed through a 0.8% agarose gel, stained with ethidium bromide, and photographed under UV exposure (15).

For control purposes, reaction mixtures containing distilled water and all other reagents but no template were included. The thermocyclers and the pipettes used for preparing the PCR reagents were kept in a different location from where gels were loaded and stained and pictures were taken. All reagents used in one experiment were taken in

TABLE 3. Nontoxigenic *V. cholerae* O1 strains negative for *ctxA* by PCR[a]

Strain	Biotype	Serotype	Origin[b]	Year of isolation
2741-80	El Tor	Inaba	Florida	1980
917-84	El Tor	Inaba	Georgia	1984
1029-84	El Tor	Inaba	Florida	1984
2452-88	El Tor	Inaba	Mississippi	1988
2483-85	El Tor	Inaba	California	1985
X316	El Tor	Inaba	Guam, South Pacific	1976
468-83[c]	El Tor	Ogawa	Philadelphia	1983
C7948	El Tor	Inaba	Hong Kong	1984
C7953	El Tor	Inaba	Hong Kong	1987
2583-87	El Tor	Ogawa	Peru	1987
2584-87	El Tor	Ogawa	Peru	1987
884-82	Atypical	Inaba	Florida	1982
2494-85	Atypical	Inaba	Florida	1985
1077-79	Atypical	Inaba	Louisiana	1979
1165-77	Atypical	Inaba	Alabama	1977
2168-81	Atypical	Ogawa	Florida (water)	1981
1074-78	Atypical	Ogawa	Brazil (sewage)	1978
2633-78	Atypical	Ogawa	Brazil (sewage)	1978

[a] Nontoxigenic strains had previously been found cholera toxin negative by ELISA and by genetic probing for the cholera toxin gene (2, 8).
[b] All samples were isolated from human stools, unless indicated otherwise.
[c] Linked to travel to Mexico.

aliquots from the freezer and discharged at the end of the day.

Samples (15 μl) of the amplified DNA were purified by using a CL6B Sephadex spin column and digested by using either *Rsa*I or *Alu*I (as described by the manufacturer, Bethesda Research Laboratories). The fragments were separated on a 0.8% acrylamide gel at 60 V for 3 h. Digesting the 564-bp *ctxA* amplicon with *Rsa*I resulted in three fragments, 480-, 70-, and 10-bp fragments (the last one is not visible on the gel). Digestion with *Alu*I resulted in two fragments, 500- and 65-bp fragments.

ELISA for cholera toxin. All *V. cholerae* strains were tested for cholera toxin production in an enzyme-linked immunosorbent assay (ELISA) which is described in detail elsewhere (2, 8).

RESULTS

Development and evaluation of the PCR test for cholera toxin subunit A gene. To develop the diagnostic PCR, we initially evaluated three different primer combinations and selected the primer set which most effectively amplified the *ctxA* gene, as determined by the intensity of the amplicons after horizontal gel electrophoresis (data not shown). Primers CTX2 and CTX3 amplified a 564-bp region of the *ctxA* gene from known toxigenic *V. cholerae* O1 control isolates.

Of 178 *V. cholerae* O1 isolates that were positive in the ELISA, 177 were initially positive by PCR (Tables 1 and 2); the one false-negative isolate was positive upon retesting, suggesting a possible problem with the first DNA extract. All 26 *V. cholerae* O1 isolates that were negative in the ELISA were also negative by PCR (Tables 1 and 3).

E. coli strains known to produce an enterotoxin (LT) structurally similar to cholera toxin were tested in the PCR for cholera toxin (data not shown). Nineteen LT-producing strains were negative by PCR. Sixteen LT-positive *E. coli* strains generated a 400-bp amplicon when primers CTX2 and CTX3 were used at an annealing temperature of 50°C. This

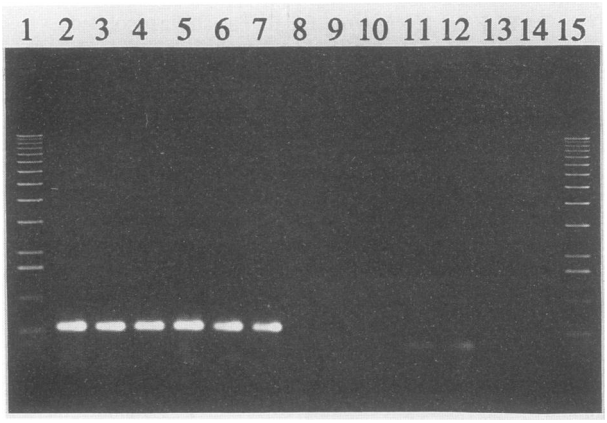

FIG. 1. Analysis of PCR products from the *ctxA* genes. Lanes: 1 and 15, molecular weight ladder; lanes 2 to 7, *V. cholerae* C7869 (lanes 2 and 5), C7870 (lanes 3 and 6), and C7871 (lanes 4 and 7) taken from transport slants (lanes 2 to 4) or cultivated on blood agar plates (lanes 5 to 7); lane 8, *C. jejuni* D121; lane 9, *A. hydrophila* A2417; lane 10, nontoxigenic *V. cholerae* O1 X316; lanes 11 to 14, LT-positive *E. coli* SSV 6547 (lanes 11 and 13) and B1 (lanes 12 and 14) amplified at 50 (lanes 11 and 12) and 60°C (lanes 13 and 14).

false-positive band was also generated by the CTX2 primer alone at 50°C. The nonspecific amplicon was not seen when the annealing temperature was adjusted to 60°C (Fig. 1). Thirty-four *A. hydrophila* and *Campylobacter* strains that were reported to produce proteins similar to cholera toxin were negative by PCR (data not shown).

The *ctxA* amplicon was produced directly from transport medium before subculture (Fig. 1), and these results corresponded 100% with amplification after cultivation from a single colony and with ELISA results (data not shown). Three different thermocyclers were used, and all performed similarly with respect to *ctxA* identification. One of the thermocyclers used a convenient 96-well microtiter plate format, and no cross contamination was observed.

Application of PCR to the Latin American cholera epidemic. Initially, suspected *V. cholerae* strains, which were sent or brought from Latin America, were serologically confirmed and assayed for cholera toxin by ELISA. After development of the PCR, all incoming isolates were first screened for the presence of *ctxA* and were subsequently confirmed by serotyping and the ELISA (Table 1). There were no discrepancies between PCR and ELISA results for these strains.

DISCUSSION

The identification of cholera toxin production is an important step in the diagnosis of cholera, because only toxin-producing strains have been associated with severe, watery diarrhea and epidemics (9). We have developed a PCR for the detection of the cholera toxin subunit A gene in *V. cholerae* strains and, after evaluation, have applied it as a part of our routine identification procedure. Shirai et al. (17) also recently described a similar PCR assay for the cholera toxin gene. This PCR assay was applied successfully to both bacterial colonies and fecal samples from patients with suspected cholera.

An important factor in evaluating any DNA-based test is the specificity of the DNA sequences chosen for the gene and strains of interest. Several different bacterial species

have been reported to possess toxins or proteins that are immunologically or genetically cross-reactive with cholera toxin (10, 12–14, 16). A nonspecific reaction (e.g., DNA amplicon of the incorrect size) was observed for some of the enterotoxigenic *E. coli* strains at low annealing temperatures. These data are consistent with the concept of an LT gene family with various degrees of relatedness (9). However, the PCR product was not homologous to *ctxA* and was eliminated by increasing the annealing temperature to 60°C. Other bacterial species reported to produce proteins that cross-react with cholera toxin in immunologic tests were tested in the PCR and found to be negative. These isolates were also negative in the cholera toxin ELISA, indicating little homology with either cholera toxin or LT. The sequences of the amplicons of correct size were verified by restriction endonuclease digest patterns, and sequence verification or hybridization with internal probe should be part of a normal control procedure in a diagnostic PCR assay.

In the evaluation of an assay, it is important to confirm that a wide selection of organisms with the characteristic under examination give uniformly positive reactions. The *V. cholerae* strains analyzed in this study were isolated worldwide during the last 60 years (2). The collection of known toxigenic and nontoxigenic control strains contained both classical and El Tor strains of Inaba and Ogawa serotypes. After evaluation with the well-characterized control strains in our study, the PCR was applied to unknown isolates sent to the Centers for Disease Control from suspected cases of cholera in Latin America during 1991 (5–7). Epidemiologic investigations of the cholera outbreaks in Latin America included isolates from possible sources of transmission; thus, strains from food and water were also assayed.

An important advantage of the PCR over the ELISA is its rapidity. During the investigation of outbreaks of cholera, the presence of *ctxA* could be determined within 4 h, while the cholera toxin ELISA required at least 4 days. Important public health decisions were made on the basis of the PCR data, and control strategies were implemented, in some situations, within 24 h. The PCR also uses commercially available reagents and protocols; the automation of the process using a thermocycler ensures high reproducibility with respect to temperature and reaction time. The use of PCR is becoming increasingly important in the diagnosis of infectious diseases (12, 17). It has the potential to be not only more rapid but also more specific and possibly more sensitive than currently available tests for the detection and identification of infectious agents. New PCR assays must be thoroughly controlled and evaluated before application to clinical specimens. We conclude that the PCR described above has proved to be a simple and fast alternative to immunologic assays that are currently employed in microbiology laboratories.

REFERENCES

1. **Almeida, R., J. D. N. Cameron, W. L. Cook, and I. K. Wachsmuth.** 1992. Vibriophage VcA-3 as an epidemic strain marker for the U.S. Gulf coast *Vibrio cholerae* O1 clone. J. Clin. Microbiol. **30**:300–304.
2. **Almeida, R. J., F. W. Hickman-Brenner, E. G. Sowers, N. D. Puhr, J. J. Farmer II, and I. K. Wachsmuth.** 1990. Comparison of a latex agglutination assay and an enzyme-linked immunosorbent assay for detecting cholera toxin. J. Clin. Microbiol. **28**:128–130.
3. **Baig, B. H.** 1986. Virulence markers of *Campylobacter*. Ph.D. dissertation. Emory University, Atlanta, Ga.
4. **Centers for Disease Control.** 1986. Toxigenic *Vibrio cholerae* O1 infections—Louisiana and Florida. Morbid. Mortal. Weekly Rep. **35**:606–607.
5. **Centers for Disease Control.** 1991. Cholera—Peru 1991. Morbid. Mortal. Weekly Rep. **40**:108–110.
6. **Centers for Disease Control.** 1991. Importation of cholera from Peru. Morbid. Mortal. Weekly Rep. **40**:258–259.
7. **Centers for Disease Control.** 1991. Cholera—New Jersey and Florida. Morbid. Mortal. Weekly Rep. **40**:287–289.
8. **Cook, W. L., K. Wachsmuth, S. R. Johnson, K. A. Birkness, and A. R. Samadi.** 1984. Persistence of plasmids, cholera toxin genes, and prophage DNA in classical *Vibrio cholerae* O1. Infect. Immun. **45**:222–226.
9. **Finkelstein, R. A.** 1988. Cholera, the cholera enterotoxins, and the cholera enterotoxin-related enterotoxin family, p. 85–102. *In* P. Owen and T. J. Foster (ed.), Immunological and molecular genetic analysis of bacterial pathogens. Elsevier Science Publisher, Amsterdam.
10. **James, C., M. Dibley, V. Burke, J. Robinson, and M. Gracey.** 1982. Immunological cross-reactivity of enterotoxins of *Aeromonas hydrophila* and cholera toxin. Clin. Exp. Immunol. **47**:34–42.
11. **Mekalanos, J. J., D. J. Swartz, and G. D. N. Pearson.** 1983. Cholera toxin gene: nucleotide sequence, deletion analysis and vaccine development. Nature (London) **306**:551–557.
12. **Olive, D. M.** 1989. Detection of enterotoxigenic *Escherichia coli* after polymerase chain reaction amplification with a thermostable DNA polymerase. J. Clin. Microbiol. **27**:261–265.
13. **Olsvik, Ø., K. Wachsmuth, G. Morris, and J. C. Feeley.** 1984. Genetic probing of *Campylobacter jejuni* for cholera toxin and *Escherichia coli* heat-labile enterotoxin. Lancet **i**:449.
14. **Ruiz-Palacios, G. M., J. Torres, N. I. Torres, E. Escamilla, B. R. Ruiz-Palacios, and J. Tamayo.** 1983. Cholera-like enterotoxin produced by *Campylobacter jejuni*. Lancet **ii**:413–416.
15. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
16. **Schultz, A. J., and B. A. McCardell.** 1988. DNA homology and immunological cross-reactivity between *Aeromonas hydrophila* cytotonic toxin and cholera toxin. J. Clin. Microbiol. **26**:57–61.
17. **Shirai, H., M. Nishibuchi, T. Ramamurthy, S. K. Bhattacharya, S. C. Pal, and Yoshifumi Takada.** 1991. Polymerase chain reaction for detection of cholera enterotoxin operon of *Vibrio cholerae*. J. Clin. Microbiol. **29**:2517–2521.
18. **Wachsmuth, I. K., C. A. Bopp, and P. I. Fields.** 1991. Difference between toxigenic *Vibrio cholerae* O1 from South America and the US Gulf Coast. Lancet **i**:1097–1098.